THE OCCURRENCE OF LICHEN PHENOLICS AND THEIR CATABOLITES IN A FREE-LIVING ALGA, *LOBOPHORA VARIEGATA* (PHAEOPHYTA)

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ABSTRACT. — *Lobophora variegata* (Lamouroux) Womersley, a free-living alga, contains several phenolics which have been, as yet, considered as lichen products, as usnic acid does. In addition, orcinol, phloroglucinol and salicylic acid have been identified by TLC, HPLC and microcrystal test.

KEY WORDS : lichen phenolics products, *Lobophora variegata*, Phaeophyta.

INTRODUCTION

Depsides, depsidones and dibenzofurans, including usnic acids, are phenols produced by the fungal partner of lichens, although the symbiosis state is always required in order to synthesize these compounds. This is in agreement with the failure in the production of these phenolics by isolated mycobionts, although there are several workers that claim to have been successful in this problem. CASTLE and KUBSCH (1949) describe that the isolated mycobiont of *Cladonia cristatella* produces usnic, didymic and rhodocladonic acids but no experimental evidences are brought forward. In addition, these results could not be duplicate for other authors (AHMADJIAN, 1980). Squamatic acid is synthesized by the cultured mycobiont of *C. crispata* after irradiation of the thallus with UV light (EJIRI & SHIBATA, 1975). Data about the synthesis of D-usnic and salazinic acids by the mycobiont of *Ramalina crassa* are extremely contradictory (KOMIYA & SHIBATA, 1969; KUROKAWA et al., 1969).

It is possible that the synthesis of these compounds was really achieved by isolated mycobionts but the products could not be detected if they were rapidly

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hydrolyzed. This is suggested by the fast hydrolysis of lecanoric acid, produced by the free-living fungus *Pyricularia* to orsellinic acid which is secondly converted into orcinol (UMEZAWA et al., 1974). Another free-living fungus, *Aspergillus terreus*, produces the depside 4-0-demethyl barbatic acid (YAMAMOTO et al., 1976), although they are, as yet, lonely cases.

The occurrence of these phenolics in algae, including both isolated and associated photobionts, has never been reported. It can only be accepted that certain amounts of these compounds can be translocated from the mycobionts, where they are produced, to the photobiont cells (AHMADJIAN & JACOBS, 1983; VICENTE et al., 1983). The role of algae in the symbiosis, required to achieve the synthesis of phenols, concerns to the supply of an inhibitor of phenolic acid decarboxylases (MOBACH & SCHULTZ, 1971), which is produced by the photobiont and translocated to the mycobionts, where it would then prevent the use of monocyclic precursors in the production of bicyclic phenols by inhibiting the action of fungal biosynthetic enzymes (CULBERSON & AHMADJIAN, 1980).

In this work, usnic acid and several single phenols have been identified in cell-free extracts of the free-living alga, *Lobophora variegata*.

MATERIAL AND METHODS

Lobophora variegata J. Ag. was collected in the Tambaú Beach (Pariba, Brazil). Dry thallus was extracted with pure methanol and analyzed by TLC and HPLC. Thin layer chromatograms were developed on Merck Silica Gel 60 plates of 16 cm height. The solvent system was benzene:dioxane:acetic acid (90:25:4 v/v), according to CULBERSON & KRISTINSON (1970). The developed plates were air-dried examined under UV light (366 nm) or sprayed with 10 % H_2SO_4 and heated at 110°C until colour developed. D-usnic acid (Laako, Finland), orcinol, salicylic and evernic acids (Sigma) and phloroglucinol (Merck) were used as standards.

Methanolic extract was also used for HPLC analysis (LEGAZ & VICENTE, 1983) in a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer. Chromatographic conditions were as follows : reverse phase column, 300 x 4 i. d., packed with micro-Pak MCH-10; mobile phase, acetic acid:water (2:98 v/v):methanol (20:80 v/v); flow rates (and pressures), 0.5 $ml.min^{-1}$ (125 atm), 1.0 $ml \cdot min^{-1}$ (164 atm) and 1.5 $ml \cdot min^{-1}$ (225 atm); temperature, 20°C; absorbance range, 0.05; detector, UV set at 254 nm; internal standard, evernic acid, 0.1 $mg \cdot ml^{-1}$. Standards were the same above described.

Microcrystal test was performed according to ASAHINA & SHIBATA (1954) by using 2 % benzidine in ethanol.

RESULTS

Analysis by TLC, using as mobile phase benzene:dioxane:acetic acid, of the methanolic extract of *L. variegata* indicates the existence of a high proportion of

phenolic compounds that develop colour with sulfuric acid after heating. Chromatographic characteristics of these compounds are shown in Table 1. Three of these substances can undubitably be identified since their mobility on thin layers, their fluorescence under UV light and colour reactions are almost identical to those found for the markers. They are phloroglucinol, orcinol and salicylic acid. A spot which shows great mobility on this layers (Rf = 0.92) coincides with usnic acid but the compound isolated from algal extract produces a strong reddish fluorescence under UV light whereas the marker clearly acts as a quencher of fluorescence. However, the formation of microcrystals with benzidine (Fig. 1) indicates that *L. variegata* contains some amount of this last phenol.

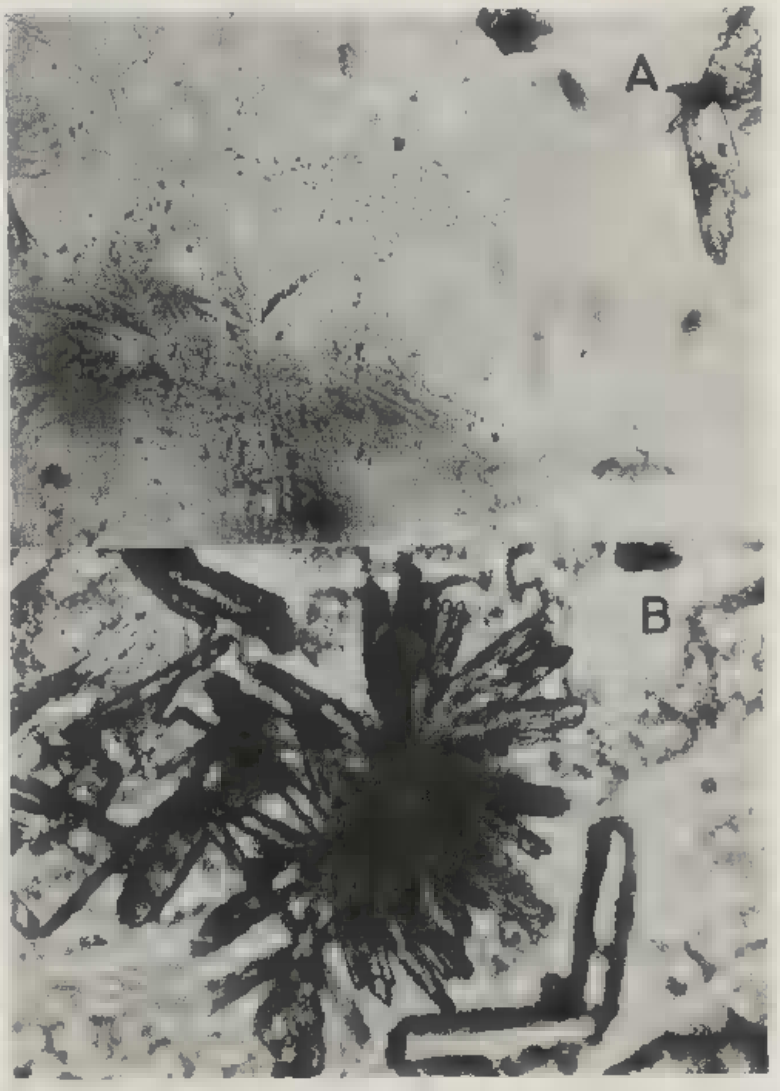

Fig. 1. – Microcrystals obtained with 2 % benzidine from a sample of D-usnic acid (A) and from the algal extract (B).

Table 1. — TLC separation of algal phenolics and standards.

Algal extract	Rf	Colour with H_2SO_4	Fluorescence
	1.0	brown	red
	0.97	brown	red
	0.95	brown	red
	0.92	pale brown	red
	0.90	violet	red
	0.83	heavy violet	red
	0.80	- - - -	blue
	0.60	- - - -	blue
	0.55	yellowish	- - - -
	0.20	yellowish	- - - -
Markers			
Usnic acid	0.92	pale brown	quencher
Salicylic acid	0.60	- - - -	blue
Orcinol	0.55	yellowish	- - - -
Phloroglucinol	0.20	yellowish	- - - -

Relative retention time of the markers in HPLC, as well as that of the internal standard, evernic acid, is summarized in Table 2. A flow rate of 0.5 $ml \cdot min^{-1}$ has been chosen because phloroglucinol and orcinol individually separate as two well defined peaks from a equimolar mixture at this flow rate value. The peak of phloroglucinol coalesces with that of orcinol when the markers are chromatographed together at a flow rate of 1.0 $ml \cdot min^{-1}$ (Fig. 2).

Table 2. — Retention time in HPLC of the phenolics used as markers

Compound	Retention time (min)	Relative retention time (min)
Phloroglucinol	4.55	0.81
Orcinol	5.06	1.32
Salicylic acid	6.16	2.42
Usnic acid	8.12	6.86

The occurrence of monocyclic phenols in the methanolic extract of *L. variegata* is confirmed in this way. Both phloroglucinol and orcinol conveniently separate (Fig. 3A) but salicylic acid shows a retention time value slightly lower than that found for the marker. Nevertheless, when the algal extract is loaded with 1.0 $mg \cdot ml^{-1}$ salicylic acid, a sole peak at 6.1 min strongly increases (Fig. 3 B).

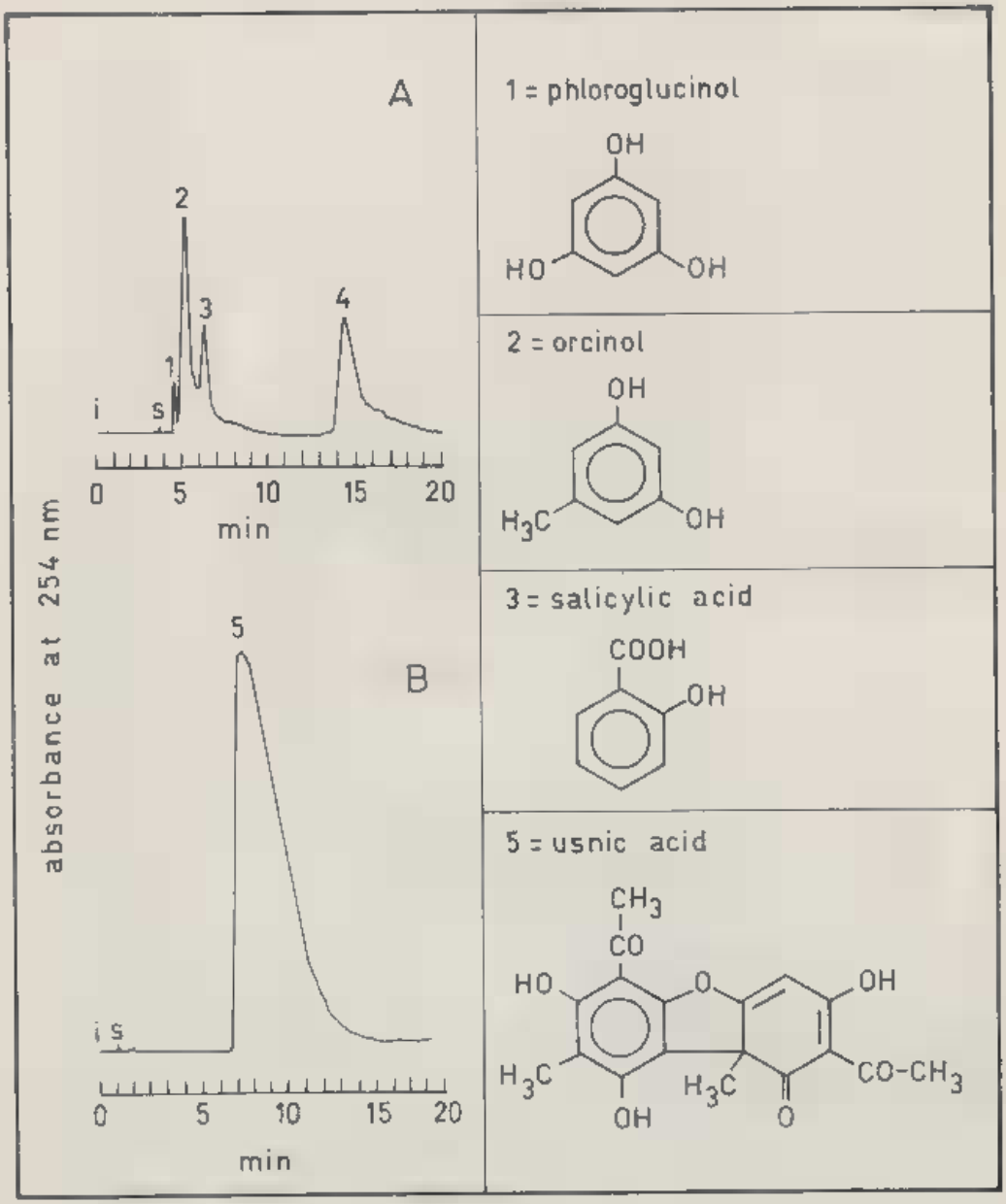

Fig. 2. — Chromatographic traces in HPLC of the different markers. In A) phloroglucinol (1), orcinol (2), salycilic acid (3) and evernic acid (4), this last one as internal standard, were chromatographed from a mixture containing 0.1 $mg \cdot ml^{-1}$ of each one at flow rate of 0.5 $ml \cdot min^{-1}$. In B), 1.0 $mg \cdot ml^{-1}$ D-usnic acid (5) was chromatographed at a flow rate of 1.5 $ml \cdot min^{-1}$. s = solvent; i = injection.

Usnic acid shows the best resolution pattern as a gaussian peak when flow rate is increased up to 1.5 $ml \cdot min^{-1}$, with a retention time value of 8.12 min (Fig. 2). Algal extract shows a minor peak with a retention time value of 8.37

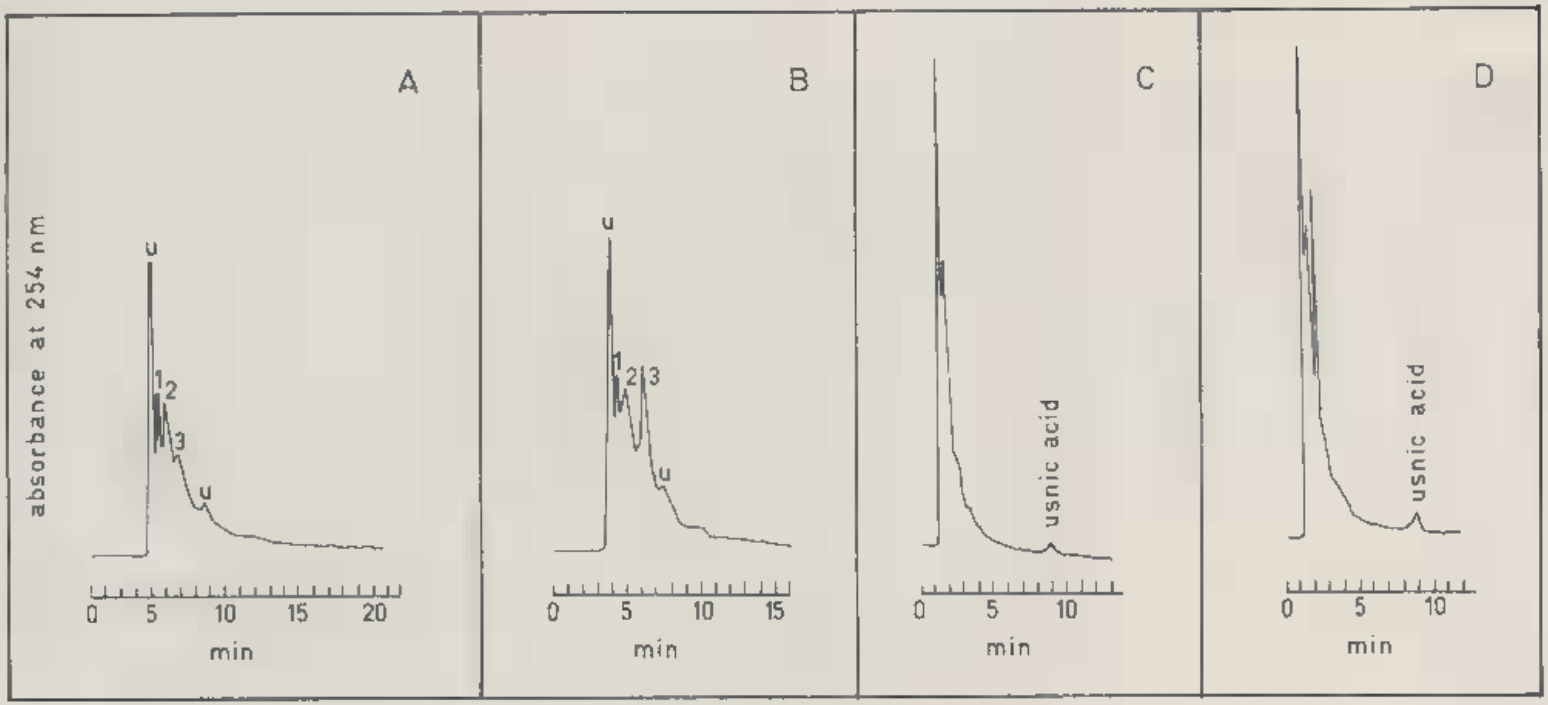

Fig. 3. — Chromatographic traces obtained by HPLC from algal extracts ■ a flow rate of 0.5 ml·min^{-1} (A and B) and 1.5 ml·min^{-1} (C and D). Two unknown substances (u), phloroglucinol (1), orcinol (2) and salicylic acid (3) are separated in A). In B), the extract was loaded with exogenous salicylic acid. Traces of usnic acid were detected in C), the peak of which selectively increased by adding exogenous D-usnic acid to the extract (D).

min (Fig. 3C), which is enhanced when the sample is loaded with 1.0 $mg \cdot ml^{-1}$ usnic acid (Fig. 3D).

DISCUSSION

Identification of three monocyclic phenolics from *L. variegata* extract, phloroglucinol, orcinol and salicylic acid, does not imply any problem in TLC. However, usnic acid is not revealed by this procedure, although a spot appears, of the Rf value of which is identical to the marker. Some doubts exist about the nature of this compound since usnic acid behaves as a quencher of fluorescence (CULBERSON & KRISTINSON, 1970) whereas the substance from *L. variegata* emits red fluorescence under UV light. It is possible that usnic acid occurs in this alga, as deduced from the microcrystal test, although this compound does not separate by TLC from another one which fluoresces. Thus, the quencher ability of usnic acid is masked. This is expected because of the high number of algal phenols which appear near the end of developed chromatogram, with Rf values from 0.9 to 1.0. This problem remains unresolved when benzene: chloroform or toluene:acetic acid are used as mobile phase (CULBERSON & KRISTINSON, 1970). However, usnic acid is perfectly separated by HPLC. Thus, its occurrence in *L. variegata* can be assured.

A secondary evidence about the occurrence of this phenolic is the presence of phloroglucinol in the methanolic extract. Usnic acids are produced from two units of methylphloroacetophenone (TAGUCHI et al., 1969), which can catabolize to phloroglucinol, although this last one can directly be produced from a 8-carbon polyketide chain through a phloroglucinolic cyclasation (CULBERSON, 1969). It is even probable that the non-identified peak by HPLC with a retention time value of 4.1 min could be a catabolite of phloroglucinol, which is enzimatically degraded by free-living fungi (WALKER & TAYLOR, 1983).

Similar catabolic origin have both orcinol and salicylic acid. These are degradative products of both orsellinic and 6-methylsalicylic acids, respectively, which are produced by the action of two different aromatic acids synthetases (GAUCHER & SHEPHERD, 1968). It has been reported that an orsellinic acid decarboxylase exists in lichenized algae (MOSBACH & EHRENSWÄRD, 1966; MOSBACH & SCHULTZ, 1971), the action of which produces orcinol from orsellinic acid, and that it plays a main role in the symbiosis lichen state (CULBERSON & AHMADJIAN, 1980) but, as yet, this has not been reported from free-living algae.

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